

Amendments to the Specification:

Please replace the paragraph beginning at page 1, line 4 with the following paragraph:

The present invention is directed to compositions of matter useful for the diagnosis and treatment of tumor in mammals and to methods of using those compositions of matter for the same. The invention further relates to the field of non-human, transgenic animal models for hepatocellular carcinoma.

Please replace the paragraph beginning at page 6, line 22 with the following paragraph:

BRIEF DESCRIPTION OF THE DRAWINGS

Please replace the paragraph beginning at page 6, line 23 with the following paragraph:

Figure 1 shows the nucleotide sequence (SEQ ID NO:1) of a cDNA containing a nucleotide sequence (nucleotides 464-1111) encoding native sequence FGF 19, wherein the nucleotide sequence (SEQ ID NO:1) is a clone designated herein as "DNA49435-1219". This clone is also designated herein as TAT49435. Also presented in bold font and underlined are the positions of the respective start and stop codons.

Please replace the paragraph beginning at page 6, line 27 with the following paragraph:

Figure 2 shows the amino acid sequence (SEQ ID NO:2) of a native sequence FGF 19 polypeptide as derived from the coding sequence of SEQ ID NO:1. Also shown are the approximate locations of various other important polypeptide domains.

Please replace the paragraph beginning at page 6, line 30 with the following paragraph:

Figures 3A-3D show preneoplastic hepatocellular changes in FGF19 transgenic mice. As early as 14 weeks of age pericentral hepatocytes formed a dense cluster around the central veins (arrows) with polarization of nuclei of the innermost hepatocytes away from the vessel lumen in FGF19 transgenics (A) which was not present in liver from non-transgenic littermate mice (B). Pericentral small dysplastic hepatocytes (C) were the predominant type of hepatocellular dysplasia although foci of large dysplastic hepatocytes (D) were occasionally noted. Arrows delineate areas of altered hepatocellular foci (shown at higher magnification in the insets). Magnification x 100 (A and B) and x 400 (C and D). Inset magnification x 400 (A and B) and x 600 (C and D).

Please replace the paragraph beginning at page 6, line 37 with the following paragraph:

~~Figures 4A-4D show hepatocellular neoplasia in FGF19 transgenic mice. (A) Multiple, large, raised tumors protrude from the hepatic surface of the liver from a 10-month-old FGF19 transgenic mouse (arrows). (B) Histologically, neoplastic cells invade and replace normal hepatic architecture and are arranged in solid sheets or cords. Arrows mark the border of the tumor and adjacent normal liver. (C) Pleomorphism of neoplastic hepatocytes and atypical mitotic figures. Magnification x 40 (B) and x 400 (C).~~

Please replace the paragraph beginning at page 7, line 3 with the following paragraph:

~~Figures 5A-5D show FGFR4 expression in murine liver. Brightfield (A) and darkfield (B) illumination of ISH with a murine FGFR4 riboprobe showing expression in perivenular and random hepatocytes. (C) and (D) Higher magnification of brightfield demonstrating silver grains over random small hepatocytes. Magnification x 100 (A and B) and x 400 (C and D).~~

Please replace the paragraph beginning at page 7, line 7 with the following paragraph:

~~Figures 6A-6E show increased proliferation of pericentral hepatocytes in FGF19 transgenics. Immunostaining for BrdU after 5-day infusion by osmotic minipump of liver from a wild-type (A) and FGF19 transgenic mouse (B). Morphometric analysis of BrdU-immunostained sections from FGF19 transgenics compared to wild-type mice: (C) 2-4 months (D) 7-9 months and (E) FGF19 injected mice. The labeling index denotes the number of BrdU-positive hepatocytes divided by the total number of cells counted and indicated as a percentage. The asterisk (*) indicates p-value less than 0.05. Magnification x 200 (A and B) and x 600 (inset in B).~~

Please replace the paragraph beginning at page 7, line 13 with the following paragraph:

~~Figures 7A-7D show glutamine synthetase immunoreactivity of dysplastic and neoplastic hepatocytes from FGF19 transgenics. (A) Neoplastic cells are strongly glutamine synthetase positive. (B) Liver from a wild-type mouse showing normal perivenular glutamine synthetase immunostaining. (C) Dysplastic hepatocytes are strongly glutamine synthetase positive. (D) Normal glutamine synthetase immunoreactivity of perivenular hepatocytes. Magnification x 40 (A and B) and x 400 (C and D).~~

Please replace the paragraph beginning at page 7, line 18 with the following paragraph:

~~Figures 8A-8E show expression of AFP by neoplastic and dysplastic hepatocytes. Increased expression of AFP mRNA in FGF19 transgenic liver compared to wild-type liver at (A)~~

~~2-4 months of age and (B) 7-9 months of age. The asterisk (*) indicates p-value less than 0.05. Brightfield (C) and darkfield (D) illumination of ISH with AFP riboprobe showing expression of AFP by pericentral dysplastic hepatocytes (arrows). Brightfield (E) and darkfield (F) illumination of ISH with AFP riboprobe showing expression of AFP by neoplastic hepatocytes (arrows). Magnification x 100.~~

Please replace the paragraph beginning at page 7, line 24 with the following paragraph:

~~Figures 9A-9D show β -Catenin immunoreactivity of neoplastic hepatocytes from FGF19 transgenics. (A) Strong nuclear staining of neoplastic cells compared with surrounding liver. Arrows mark the border of the tumor and adjacent normal liver. (B) Neoplastic hepatocytes with nuclear immunoreactivity for β -catenin. Magnification x 200 (A) and x 400 (B). (C) Amino acid sequence alignment of the N-terminal region of β -catenin from wild-type (top) and mutant clones with amino acid substitutions (bold) in and adjacent to the GSK-3 β phosphorylation domain (red). (D) Sequencing data for DNA from normal liver and HCC with nucleotide substitutions at codon 34 (shaded). ENBbu~~

Please replace the paragraph beginning at page 92, line 11 with the following paragraph:

³³P-labeled murine FGFR4 and AFP riboprobes were used to evaluate gene expression in murine liver, lung, spleen, kidney, and brain. To generate the probes, PCR primers were designed to amplify either a 654 bp fragment of murine AFP spanning from nt 731-1385 of NM_007423 (upper- 5' CCTCCAGGCA ACAACCATTA T (SEQ ID NO:1) and lower- 5' CCGGTGAGGT CGATCAG (SEQ ID NO:2)) or a 170 bp fragment of murine FGFR4 spanning from nt 327-497 of NM_008011 (upper- 5' CGAGTACGGGGTTGGAGA (SEQ ID NO:3) and lower- 5' TGCTGAGTGTCTTGGGGTCTT (SEQ ID NO:4)). Primers included extensions encoding 27-nucleotide T7 or T3 RNA polymerase initiation sites to allow in vitro transcription of sense or antisense probes, respectively, from the amplified products. Sections were deparaffinized, deproteinized in 4?g/ml of proteinase K for 30 minutes at 37°C, and further processed for *in situ* hybridization as previously described. ENRfu⁴³ ³³P-UTP labeled sense and antisense probes were hybridized to the sections at 55°C overnight. Unhybridized probe was removed by incubation in 20?g/ml RNase A for 30 minutes at 37°C, followed by a high stringency wash at 55 °C in 0.1 X SSC for 2 hours and dehydration through graded ethanols. The slides were dipped in NBT2 nuclear track emulsion (Eastman Kodak), exposed in sealed plastic slide boxes containing desiccant for 4 weeks at 4 °C, developed and counterstained with hematoxylin and eosin.

Please replace the paragraph beginning at page 93, line 33 with the following paragraph:

DNA extracted from tumor tissue was PCR amplified using forward and reverse primers 5' TAC AGG TAG CAT TTT CAG TTC AC 3' (SEQ ID NO:5) and 5' TAG CTT CCA AAC ACA AAT GC 3' (SEQ ID NO:6), respectively. PCR products were subcloned into pCR2.1 using the TA cloning kit (Invitrogen). Sequencing of subcloned PCR products was done as outlined in the ABIPRISM™BigDye™ Terminator Cycle Sequencing Ready Reaction Kit on an Applied Biosystems PRISM 3700 DNA Analyzer. M13 primers were used for the TA vector. The trace files were edited and aligned using Sequencher™ (Gene Codes Corp., Ann Arbor MI, USA). Mutations were identified by comparing the trace files to the murine β -catenin sequence published in Genbank (NM_007614).

Please replace the paragraph beginning at page 94, line 7 with the following paragraph:

As early as 2-4 months of age hepatocytes adjacent to central veins formed a single columnar row with nuclei polarized away from the endothelial basement membrane of the central vein (Fig. 3A) which was not observed in wild-type mice (Fig. 3B). Dysplastic changes (areas of altered hepatocellular foci) preceded tumor formation and were evident by 7-9 months. Within this age group, 33% of female and 7% of male transgenics had hepatocellular dysplasia without evidence of neoplasia. Interestingly, dysplastic foci were predominantly of the small cell type and oriented around central veins (Fig. 3C). Rarely, foci of large dysplastic hepatocytes were noted (Fig. 3D). FGF19 transgenic mice developed liver tumors by 10-12 months of age at an overall frequency of 53%. Within the 10-12 month-old group, 80% (8/10) of female and 22% (2/9) of male FGF19 transgenic mice had locally invasive hepatocellular carcinomas (Fig. 4). Tumors were solitary or multifocal, involving different liver lobes. Mean liver weights in the 10-12 month-old female FGF19 transgenics were increased 30% relative to liver weights of wild-type mice (mean liver weights = 1.97 and 1.54 grams, respectively; $p < 0.01$) attributable to tumor mass. The mean liver weight for 10-12 month-old male FGF19 transgenic mice was not significantly different than wild-type mice, likely due to the low incidence of tumors in the male transgenic mice (mean liver weights = 1.53 and 1.63 grams, respectively; $p = 0.37$). Histologically, neoplastic hepatocytes invaded and replaced adjacent normal hepatic parenchyma (Fig. 4B). Hepatocellular carcinomas in the FGF19 transgenic mice were predominantly the solid type although a trabecular pattern was occasionally noted. Figure 4C shows the typical morphology of neoplastic hepatocytes: neoplastic cells with nuclear pleomorphism and frequent mitoses (arrows Fig. 4C). The tumors did not metastasize. Other tissues evaluated histologically

included: lungs, heart, spleen, kidneys, bone (femur), intestines, brain, pituitary gland, thyroid glands, and skeletal muscle. Despite the fact that FGF19 was expressed in the skeletal muscle, no histologic changes were evident in that tissue.

Please replace the paragraph beginning at page 94, line 37 with the following paragraph:

FGF19 was previously shown to selectively bind with high affinity to FGFR4.^{ENRf¹⁴} Although FGFR4 expression has been demonstrated in mouse and rat hepatocytes, *in situ* hybridization with a ³³P-labeled murine FGFR4 riboprobe was used to determine expression patterns in wild-type and FGF19 transgenic mice. In both wild-type and FGF19 transgenic mice, a strong signal for murine fgfr4 mRNA was present in hepatocytes adjacent to central veins and in random, small hepatocytes throughout the lobule (Fig. 5). There was not a significant difference in signal intensity or distribution based on genotype. In addition, real-time RT-PCR did not demonstrate any difference in levels of fgfr4 mRNA between FGF19 transgenic and wild-type mice.

Please replace the paragraph beginning at page 95, line 6 with the following paragraph:

Constitutive hepatocellular proliferation is considered a prerequisite for neoplastic transformation. Therefore, *in vivo* BrdU labeling in the FGF19 transgenic mice was used to assess hepatocellular proliferation. Labeled hepatocytes were predominantly perivenular (Fig. 6B) whereas BrdU-labeled hepatocytes were rare in wild-type mice (Fig. 6A). By 2-4 months of age the BrdU labeling index of hepatocytes was eight-fold higher in FGF19 transgenic females than age matched wild-type females ($p = .00003$) and two- to three-fold higher in FGF19 transgenic males than age matched wild type males ($p = .040$) (Fig. 6C). The labeling index is also increased two- to three-fold in 7-9 month old female and male FGF19 transgenics relative to their respective controls ($p=.0000002$ and $p=.006$, respectively) (Fig. 6D). Together these data indicate hepatocellular proliferation precedes tumor development and that the proliferative fraction is predominantly pericentral hepatocytes.

Please replace the paragraph beginning at page 95, line 15 with the following paragraph:

To determine whether hepatocellular proliferation was due to acute effects of FGF19 *in vivo*, the purified protein was injected into non-transgenic female mice while infusing BrdU over 6 days. Mice receiving rFGF19 protein had a significantly higher BrdU labeling index than mice receiving vehicle alone ($p = 0.014$). Similar to results described above in FGF19 transgenic mice,

rFGF19-injected mice have a three- to five-fold increase in hepatocellular proliferation relative to vehicle-injected mice (~~Fig. 6E~~).

Please replace the paragraph beginning at page 95, line 21 with the following paragraph:

Glutamine synthetase is a marker for tracing hepatocellular lineage during preneoplastic and early neoplastic stages. In the 10-12 month old mice, 10 out of 19 FGF19 transgenic mice had HCCs. All of the FGF19 induced tumors were strongly positive for glutamine synthetase by IHC (~~Fig. 7A~~). In contrast, liver from wild-type mice showed the expected pattern of staining one to three cell layers of pericentral hepatocytes (~~Fig. 7B and 7D~~). Foci of large dysplastic hepatocytes were also glutamine synthetase positive (~~Fig. 7C~~). Glutamine synthetase immunoreactivity of the neoplastic cells suggests they originated from the one to three cell layers of hepatocytes around the central veins that constitutively express glutamine synthetase.

Please replace the paragraph beginning at page 95, line 28 with the following paragraph:

AFP is an oncofetal protein expressed by neoplastic hepatocytes but not normal adult hepatocytes and is used as an indicator of neoplastic transformation in the liver. Real-time RT-PCR showed hepatic AFP mRNA was elevated in FGF19 transgenic mice relative to wild types (~~Fig. 8A and 8B~~). At 2-4 months of age female transgenics had a thirteen-fold increase ($p=.01$) and male transgenics had an eighteen-fold increase ($p=.005$) in AFP expression relative to respective wild-type controls. The 7-9 month old transgenic females had a four-fold increase ($p=.01$) and males had a three-fold increase ($p=.03$) in AFP expression relative to respective wild type controls. Subsequently, AFP expression was evaluated by *in situ* hybridization to determine which cells were expressing AFP prior to tumor formation. Consistent with previous findings that indicated initial involvement of pericentral hepatocytes, AFP expression was demonstrated in hepatocytes adjacent to central veins (~~Fig. 8C and 8D~~). Neoplastic hepatocytes also consistently expressed AFP (~~Fig. 8E and 8F~~).

Please replace the paragraph beginning at page 96, line 8 with the following paragraph:

To further evaluate the molecular pathogenesis of HCCs in FGF19 transgenic mice, immunohistochemical staining for β -catenin in addition to cloning and sequencing exon 2 of the *β -catenin* gene from tumor tissue was used. HCCs from nine different FGF19 transgenic mice were evaluated for immunoreactivity to β -catenin antibody. Four of the nine tumors (44%) had nuclear and cytoplasmic staining for β -catenin in neoplastic hepatocytes (~~Fig 9A and 9B~~). All four tumors with β -catenin immunoreactivity were from female FGF19 transgenic mice in the 10-

12 month age group. Cloning and sequencing hepatic DNA encoding exon 2 of β -catenin from tumor tissue that was IHC positive revealed point mutations that resulted in amino acid substitutions (Fig. 9C). Overall, 16% of the clones contained mutations. A \rightarrow G or G \rightarrow A transitions were the most common mutations observed and involved codons 23, 34, 72, 76, and 80. Other transition mutations included C \rightarrow T (codon 44), T \rightarrow C (codon 70), and A \rightarrow T (codon 56). Four of the clones from three different animals had mutations within the glycogen synthase kinase-3B (GSK-3B) phosphorylation domain at codon 34 and codon 44 (Fig. 9C and 9D). Of the 4 mutations within the phosphorylation domain, 3 resulted in substitution of an amino acid with a nonpolar side chain by an amino acid with a polar uncharged (Pro45Ser) or a polar charged (Gly34Glx) amino acid side chain. The fourth amino acid substitution within the phosphorylation domain retained the polar side chain but replaced a relatively small amino acid with a larger, space-occupying molecule (Gly34Ile). Seven other mutations resulted in amino acid substitutions in regions adjacent to the GSK-3B phosphorylation domain (Fig. 9C). Of the mutations outside the phosphorylation domain, amino acid substitutions resulted in altered charge (Gln72Arg, Gln76Arg, Asx56Val), polarity (Ala80Ser, Ser23Gly) or molecular size (Phe70Leu). Mutations that affect GSK-3B phosphorylation of β -catenin prevent ubiquitination and degradation, resulting in cytoplasmic accumulation and nuclear translocation of β -catenin, which accounts for the immunoreactivity observed in this study. Figure 9C shows the amino acid alignment of all mutant β -catenin clones compared to the wild-type sequence, depicting relative positions of amino acid substitutions and the GSK-3B phosphorylation domain.

Please enter the sequence listing submitted herewith into the specification of this application.